Effect of benzene and its closed ring metabolites on intrachromosomal recombination in *Saccharomyces cerevisiae*

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Abstract

Genome rearrangements, such as DNA deletions, translocations and duplications, are associated with cancer in rodents and humans, and clastogens are capable of inducing such genomic rearrangements. The clastogen benzene and several of its toxic metabolites have been shown to cause cancer in animals. Benzene is associated with leukemia and other blood related disorders in humans. Benzene and metabolites tested negative in short-term bacterial mutation assays such as the *Salmonella* Mutagenicity Test and the *Escherichia coli* Tryptophan Reversion Assay. These assays, while reliable for the detection of point-mutagenic carcinogens, are incapable of detecting DNA strand break inducing xenobiotics. The yeast DEL assay is based on intrachromosomal recombination events resulting in deletions and is very sensitive in detecting DNA strand breaks. In previous results the DEL assay detected 17 Salmonella positive as well as 25 Salmonella negative carcinogens [Bishop, Schiestl, Hum. Mol. Genet. 9 (2000) 2427–2434]. The carcinogen benzene and its metabolites including phenol, catechol, *p*-benzoquinone and hydroquinone induced DEL recombination. The benzene metabolite 1,2,4-benzenetriol was negative. Interestingly, *p*-benzoquinone induced DEL recombination at a dose 300-fold lower than any of the other metabolites, suggesting that it might be responsible for much of benzene's genotoxicity. In addition, an excision repair deficient strain was used, but no difference was detected compared to the wildtype, indicating that DNA adducts subject to excision repair were not formed by benzene or its metabolites.

Keywords: Yeast DEL assay; Recombination; Benzene metabolites

1. Introduction

Carcinogenesis is a multistage process where genetic events, including mutations and chromosome rearrangements such as DNA deletions, result in the

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neoplastic transformation of normal cells. Here we used an assay for intrachromosomal recombination between two tandemly repeated DNA sequences resulting in deletions in yeast. The recombination substrate consists of a direct duplication of two non-functional *his3* alleles, separated by the *LEU2* gene in which one *his3* allele is deleted at the 3′ end and the other at the 5′ end [1]. Intrachromosomal recombination events between approximately 400 bp of homologous DNA fragments produce a *his3*$^+$ allele, resulting in deletion (DEL recombination) of the intervening *LEU2* gene reconstituting a *HIS3*$^+$ allele and allowing growth on *HIS3*$^-$ media.

Both Salmonella Mutagenicity Test (SMT) positive and SMT negative carcinogens including benzene are able to induce the formation of *HIS3*$^+$ recombination events in the yeast DEL assay [2–5]. Furthermore, both classes of carcinogens including benzene induce DEL recombination between the two copies of an internal duplication of the HPRT gene in human cells [6] and between two copies of the pun gene in vivo in mice [5,7,8].

The bacterial strains used in the *Salmonella* assay carry a deletion in uvrB required for error free excision repair of DNA [9,10], allowing DNA adducts which would normally be quickly repaired to persist and lead to mutation. Therefore, to enhance sensitivity of the assay a DEL recombination tester strain deficient in the excision repair gene RAD2 was used [11]. As expected, the detection of several SMT positive carcinogens is enhanced in this DNA repair deficient yeast strain. Northpoth et al. detected N7-phenylguanine in the urine of benzene-exposed rats and hypothesized that these adducts were formed through the reaction of benzene-epoxide with guanine residues and that its presence in the urine was the result of the excision repair of this DNA adduct [12]. If so, the excision repair deficient yeast strain should be more sensitive to benzene induced DNA deletions and toxicity, which was tested in this study.

Benzene is a human carcinogen [13]. Although the effects of low level exposure to benzene in humans remains controversial, exposure of the general population to benzene is highly regulated because of its association with acute myeloid leukemia (AML), and other blood related diseases [14–16]. Benzene is positive in both sexes and both species, mice and rats, in long-term rodent carcinogenesis tests [17]. Among benzene metabolites tested in long-term carcinogenicity assays phenol is negative, catechol is positive, and hydroquinone positive [17]. Benzene is also mutagenic in vivo [18]. In vitro studies show that benzene is mutagenic in the mouse lymphoma assay in the presence of S9 but no DNA adducts are seen even at mutagenic levels [19]. However, benzene causes oxidative damage. In the presence of S9, benzene induces DNA strand breaks [20] and is clastogenic [21]. Benzene induces gene duplicating but not gene-inactivating mutations at the glucophorin A locus in exposed humans which has been explained by recombination or genome rearrangement [22]. Benzene, however, lacks mutagenic activity in the Salmonella assay and in other bacterial assays [23,24]. Thus, benzene and its metabolites primarily cause clastogenicity, as opposed to the induction of single or a few base pair changes, and produce negative or equivocal results in bacterial reverse mutation assays.

It is believed that metabolism of benzene is required for its toxic activity [25–28]. Benzene is metabolized to phenol, hydroquinone, catechol and 1,2,4-benzenetriol and the hydroquinone and catechol can be further metabolized to 1,4-(or *p*)benzoquinone and 1,2-benzoquinone [25,27,29–35]. Some investigators claim that these reactive quinones are responsible for benzene-induced toxicity [28,36,37]. Since the yeast DEL recombination assay detected benzene as positive, we investigated the effects and relative potency of its closed ring metabolites including phenol, catechol, *p*-benzoquinone, hydroquinone and 1,2,4-benzenetriol. We also used a DEL recombination tester strain deficient in RAD2 to investigate whether benzene or its metabolites cause DNA damage subject to nucleotide excision repair.

2. Materials and methods

2.1. Strains

The diploid yeast strain RS112 (*MATa/α ura 3-52/ura3-52 leu2-3,112/leu2-Δ98 trp5-27/TRP5 ade2-40/ade2-101 ilv1-92/ILV1 HIS3::pRS6/his3-Δ200 LYS2-/lys2-801*) was used [3,4]. RS112 contains the DEL system at one *HIS3* chromosomal locus, located on chromosome VIII, while the other chromosome is deleted for the entire *HIS3* locus. The strain RS177

[38] is isogenic to RS112 except for a genomic deletion of the *RAD2* gene (*rad2::URA3/rad2::URA3*).

2.2. Media

(SC-LEU) Medium lacks the amino acid leucine and was used for the pre-growth of yeast cultures and the overnight exposure to test compound [39]. SC-HIS Medium lacks the amino acid histidine and was used to wash the cells following the overnight exposure to test compound [39]. SC-HIS Medium with pH Indicator and Synthetic Complete Medium with pH Indicator were used in the plating of cells and were prepared as described by Sommers et al. [38]. All media used were obtained from Xenometrix Inc. (Boulder, CO).

2.3. Chemicals

Benzene (>99%) (CAS No. 71-43-2), catechol (>99%) (CAS No. 120-80-9), hydroquinone (>99%) (CAS No. 123-31-9), *p*-benzoquinone (>99%) (CAS No. 106-51-4), phenol (>99%) (CAS No. 108-95-2), were purchased from Sigma Chemical Co. (St. Louis, MO), Acetone was used as a solvent in these experiments and was obtained from Sigma Chemical Co. Ethyl methanesulfonate, used as a positive control compound, was obtained from Sigma Chemical Company (St. Louis, MO). All compounds were dissolved in acetone, which was used in the assay at a final concentration of 2%.

2.4. Experimental procedure

The yeast strains RS112 and RS177 were pre-grown in SC-LEU medium as previously described [3,38]. RS112 and RS177 were tested in parallel from the same test compound stock solution wherever possible. Ethyl methanesulfonate at a concentration of 2 mg/ml was used as a positive control for each experiment. Approximately $1–2 \times 10^6$ cells were then exposed to test compound for approximately 17 h.

The microtiter plate assay procedure of Sommers et al. [38] was used to determine recombination frequency, survival relative to the untreated controls, and the number of generations during exposure to test compound. Following exposure to the test compound, cells were pelleted and resuspended in SC-HIS medium to an optical density (600 nm) of approximately 0.1 (1×10^6 cells/ml) and diluted into 40 ml SC-HIS Medium with pH Indicator or 40 ml SC Complete Medium with pH Indicator. The medium containing yeast was then transferred into 384 well microtiter plates (Nunc) (100 μl/well). The microtiter plates were placed in locking plastic bags to prevent evaporative loss of medium and incubated for 3 days at 30 °C. After incubation the microtiter plates and the fraction of wells, both with and without yeast colonies, was determined. Presence of yeast was verified by color change of the pH Indicator and formation of colonies within the wells.

The recombination frequency is defined as the number of recombinants divided by the number of viable cells, or, in this case, the number of recombinants or viable cells per microtiter plate at appropriate dilutions, as predicted by poisson distribution [11,38]. The recombination frequency is therefore defined by the equation $-\log_n$ [(total no. of wells – growth positive wells)/total wells] as previously described [11,38]. Gen-Tox Software Version 1.3 (Xenometrix Inc., Boulder, CO) was used to calculate recombination frequencies and relative survival.

2.5. Statistical analysis

Each experiment was conducted independently three times. Statistical significance was determined using Student's *t*-test ($n=3$, $\alpha=0.05$), where values were calculated using the descriptive statistics functions of Microsoft Excel 2000 (Microsoft Inc., Redmond, WA). Analysis of Covariance was completed using Statistical Analysis Software Version 8 (SAS Institute, Cary NC).

3. Results

Benzene and its closed ring metabolites; phenol, catechol, *p*-benzoquinone, hydroquinone and 1,2,4-benzenetriol were tested for the ability to induce intrachromosomal recombination in *S. cerevisiae*. The mean spontaneous recombination frequency for RS112 colonies used in the experiment was 1.95 (± 0.25) $\times 10^{-4}$. The mean spontaneous recombination frequency for strain RS177 was 2.73 (± 0.12) $\times 10^{-4}$, which was significantly higher than that for RS112 as determined by Student's *t*-test ($n=6$, $\alpha=0.05$). The

Table 1
Effects of benzene and benzene metabolites on DEL recombination in strain RS112

Benzene (mg/ml)	0	2.5	5.0	7.5	10.0	12.5
Recombination frequency (10^{-4})	1.15±	1.35	1.02	1.36	2.65[a]	5.77[a]
S.D.	±0.29	±0.31	±0.51	±0.25	±0.93	±0.42
Fold induction	1.0	1.2	0.85	1.22	2.28	5.23
% Survival	100	98.8	93.5	80.5	31.9	8.20
Phenol (mg/ml)	0	0.5	1.0	2.5	5.0	
Recombination frequency (10^{-4})	2.46	2.04	2.84	5.86[a]	8.79[a]	
S.D.	±0.44	±0.30	±0.61	±1.46	±1.21	
Fold induction	1.0	0.84	1.15	2.51	3.61	
% Survival	100	97.8	78.7	25.6	5.85	
Catechol (mg/ml)	0	1.0	2.0	3.0	4.0	5.0
Recombination frequency (10^{-4})	1.91	1.98	3.10	4.07[a]	6.63[a]	8.94[a]
S.D.	±0.40	±0.46	±0.33	±0.10	±0.80	±0.66
Fold induction	1.0	1.04	1.62	2.13	3.47	4.68
% Survival	100	96.5	94.7	82.7	78.2	58.8
p-Benzoquinone (mg/ml)	0	0.005	0.01	0.015	0.02	0.025
Recombination frequency (10^{-4})	1.70	1.36	2.15	5.02[a]	20.1[a]	23.1[a]
S.D.	±0.06	±0.11	±0.28	±0.57	±0.60	±7.2
Fold induction	1.0	0.81	1.27	2.96	11.9	13.6
% Survival	100	99.0	88.7	58.4	27.0	10.1
Hydroquinone (mg/ml)	0	5.0	6.0	7.0	8.0	9.0
Recombination frequency (10^{-4})	2.51	5.36[a]	5.57[a]	7.12[a]	7.65[a]	8.67[a]
S.D.	±0.21	±0.80	±0.24	±0.43	±0.85	±0.96
Fold induction	1.0	2.13	2.23	2.84	3.05	3.45
% Survival	100	62.5	49.2	46.6	44.1	43.1

[a] Statistical significance of the *HIS3*$^+$ recombination frequencies relative to control values was determined using a Student's *t*-test ($n=3$, $\alpha=0.05$).

higher spontaneous reversion frequency for RS177 is due to the presence of the *rad2Δ* nucleotide excision repair defect as noted previously [11]. It should also be noted that intrachromosomal recombination in *S. cerevisiae* is not elevated by reductions in cell viability alone, e.g., cell death does not trigger false positive responses in this assay [11,38,40].

Increase in the frequency of DEL recombination by benzene and the test compound's effect on cell viability was consistent with results obtained in previous studies [38]. In strain RS112, benzene increased the recombination frequency from 1.2×10^{-4} to 2.7×10^{-4} and cell viability decreased to 32% at a concentration of 10 mg/ml. The recombination frequency increased to 5.8×10^{-4}, and viability decreased to 8.2%, at 12.5 mg/ml (Table 1). When benzene was tested in strain RS177, there was no significant difference in the recombination frequency obtained between strains RS112 and RS177. In strain RS177, benzene-induced increase in the frequency of DEL recombination was only statistically significant at the highest concentration, because this strain had higher spontaneous recombination frequency (Table 2). The high benzene concentrations required to induce recombination in the assay may be due to the high volatility of benzene [38].

Phenol produced a statistically significant increase in DEL recombination frequency in RS112 from 2.5×10^{-4} to 5.9×10^{-4} at a concentration of 2.5 mg/ml and 8.8×10^{-4} at 5.0 mg/ml, cell viability decreased to 26 and 5.9% at 2.5 mg/ml and 5.0 mg/ml, respectively (Table 1). Phenol produced a statistically significant increase in DEL recombination frequency of RS177 from 3.0×10^{-4} to 9.5×10^{-4} at 5.0 mg/ml and caused a decrease of cell viability to 8.8%. As with benzene, the increased spontaneous recombination frequency of RS177 resulted in a statistically significant increase in recombination frequency only at the highest (5.0 mg/ml) phenol concentration used.

Catechol increased the DEL recombination frequency in RS112 from 1.9×10^{-4} (0 mg/ml) to

Table 2
Effects of benzene and benzene metabolites on DEL recombination in strain RS177 (RS112 *rad2Δ*)

Benzene (mg/ml)	0	2.5	5.0	7.5	10.0	12.5
Recombination frequency (10^{-4})	2.38	2.50	2.38	2.72	3.07	6.5[a]
S.D.	±0.43	±0.47	±0.48	±0.467	±0.73	±0.79
Fold induction	1.0	1.05	1.00	1.14	1.29	2.73
% Survival	100	96.2	89.5	75.3	42.6	13.4
Phenol (mg/ml)	0	0.5	1.0	2.5	5.0	
Recombination frequency (10^{-4})	3.04	2.84	2.79	4.73	9.45[a]	
S.D.	±0.18	±0.26	±0.36	±0.46	±1.62	
Fold induction	1.0	0.93	0.91	1.56	3.11	
% Survival	100	90.3	84.4	40.2	8.80	
Catechol (mg/ml)	0	1.0	2.0	3.0	4.0	5.0
Recombination frequency (10^{-4})	2.76	2.54	3.54	3.88	6.67[a]	8.74[a]
S.D.	±0.63	±0.18	±0.49	±0.68	±0.63	±0.64
Fold induction	1.0	0.92	1.28	1.41	2.42	3.12
% Survival	100	88.1	90.3	86.7	76.0	50.4
p-Benzoquinone (mg/ml)	0	0.005	0.01	0.015	0.02	0.025
Recombination frequency (10^{-4})	2.53	2.79	2.92	5.71[a]	14.7[a]	21.5[a]
S.D.	±0.15	±0.37	±0.62	±0.58	±2.15	±3.78
Fold induction	1.0	1.10	1.16	2.26	5.81	8.50
% Survival	100	93.6	85.8	66.9	23.7	9.04
Hydroquinone (mg/ml)	0	5.0	6.0	7.0	8.0	9.0
Recombination frequency (10^{-4})	2.94	4.20	5.69[a]	6.22[a]	7.55[a]	7.73[a]
S.D.	±0.37	±1.14	±0.64	±0.57	±0.65	±0.88
Fold induction	1.0	1.43	1.94	2.12	2.57	2.63
% Survival	100	79.4	43.8	47.6	40.3	39.5

[a] Statistical significant increase of the *HIS3*$^+$ recombination frequencies relative to control values was determined using a Student's *t*-test ($n=3$, $\alpha=0.05$).

4.1×10^{-4} (3.0 mg/ml), 6.6×10^{-4} (4.0 mg/ml) and 8.9×10^{-4} (5.0 mg/ml). Unlike benzene and phenol, recombination was induced at test compound concentrations that yielded more than 50% viable cells (Table 1). Catechol produced a statistically significant increase in DEL recombination in RS177 at the two highest concentrations tested, 4.0 and 5.0 mg/ml (Table 2).

p-Benzoquinone, a topoisomerase inhibitor [41,42] increased the frequency of DEL recombination at the lowest concentration compared to other compounds. *p*-Benzoquinone was able to significantly increase DEL frequencies in RS112 and RS177 at 0.015, 0.02 and 0.025 mg/ml (Tables 1 and 2).

Hydroquinone, however, induced DEL recombination in both RS112 and RS177 at cell viabilities typically above 50% (Tables 1 and 2). The same response pattern was observed in both yeast strains and in each replicate of the experiment.

1,2,4-Benzenetriol, which was tested only in strain RS112, did not induce DEL recombination, or reduce cell viability at concentrations up to 10 mg/ml (data not shown).

4. Discussion

Benzene and its metabolites phenol, catechol, *p*-benzoquinone and hydroquinone induced DEL recombination. Benzene is a known human and rodent carcinogen [13–17]. It is believed that metabolism of benzene is required for its toxic activity [25–28]. In previous experiments, the metabolites by themselves failed to reproduce benzene's genotoxicity [43,44]. In contrast, a combination of the metabolites phenol, hydroquinone and catechol seemed to act synergistically to reproduce benzene-like induction of oxidative DNA damage and genotoxicity [19,45]. Thus, it is

interesting that in the present study these metabolites alone were able to cause DEL recombination in yeast.

The relative potency of the compounds for long-term carcinogenesis assays is benzene > catechol > hydroquinone > phenol [17]. While these compounds produce equivocal results in bacterial reverse mutation assays, they have been shown to produce chromosomal aberrations in mammalian cells both in vivo and in vitro [46–49]. Erexson et al. [46] found the relative potencies of the benzene metabolites to be catechol > *p*-benzoquinone > hydroquinone > phenol > benzene for the ability to induce Sister Chromatid Exchange (SCE) in human lymphocytes. Yager et al. [47] found hydroquinone to be the most potent of the metabolites in producing micronuclei in human lymphocytes. *p*-Benzoquinone was found to produce DNA strand breakage in human lymphocytes using the Comet Assay [48,49].

In this work, we showed that benzene and its metabolites are able to produce DEL recombination, in *S. cerevisiae*. The relative potencies (recombination frequency versus concentration) of these compounds were *p*-benzoquinone > phenol > catechol > hydroquinone > benzene. The relative potency of the compounds to cause DEL recombination most closely relates to their ability to cause SCE. When comparing the DEL recombination data with cancer-induction, phenol and benzene potencies are reversed.

Interestingly, *p*-benzoquinone induced DEL recombination at a dose 300-fold lower than phenol, the second most potent metabolite. It has been proposed that while other metabolites act through induction of reactive oxygen species, benzoquinone directly reacts with DNA to form DNA adducts [50]. Also, benzoquinone was the most potent metabolite in the comet assay using human peripheral blood mononuclear cells [36]. In fact, several investigators claim that the reactive quinones are responsible for benzene-induced toxicity [28,36,37]. This is consistent with the fact that *p*-benzoquinone was highly potent in the DEL assay.

Detection of several SMT positive carcinogens is enhanced in a DNA excision repair deficient yeast strain [11]. The detection response of the benzene metabolites was partially obscured when the DNA excision repair deficient yeast strain, RS177, was utilized. This can be attributed to the higher spontaneous recombination frequency in the DNA repair deficient strain, which resulted in lower fold induction values in a response to the test compound. When the actual recombination rates were compared for a given test compound in the two strains, normalized by the negative control rate, no difference in heterogeneity of slope was observed for any of the five benzene metabolites as determined by ANCOVA [51]. Results were also negative, as determined by ANCOVA, when relative survival rates were compared, indicating that DNA adducts recognized by nucleotide excision repair were not formed by benzene or its five metabolites.

SMT positive carcinogens usually induce recombination in a linear dose–response manner, starting at doses, which are 100–1000-fold below the lowest dose that results in cell death [52]. On the other hand, SMT negative carcinogens, including benzene, induced DEL recombination with a threshold below which no effect was detected, and the first DEL recombinagenic dose was also the first dose at which cell killing was observed [38,52]. The property of benzene inducing DEL recombination only at toxic doses is also shared by its metabolites (Table 1). These data indicate that DEL recombination is inducible by DNA damage that is simultaneously cytotoxic. One primary candidate is DNA strand breaks. In fact, DNA double-strand breaks (DSB) are the most potent inducers of DEL recombination [53,54]. The primary pathway to repair DNA DSBs in yeast is homologous recombination versus nonhomologous end joining in mammalian cells, but both are inducible by DNA DSBs. Thus, a DSB that induces DEL recombination in yeast could induce DNA deletions or translocation in mammalian cells that may be involved in carcinogenesis.

Oxidative stress induces DNA strand breaks [55] that may in turn induce DNA deletions. A yeast strain mutated for *SOD1* and *SOD2*. both encoding different forms of superoxide dismutase, which is responsible for dismutation of the superoxide anion to hydrogen peroxide and water, was many times more sensitive to benzene than the isogenic wildtype strain [56]. Furthermore, benzene treatment also caused an increase in free radical formation detected by dichlorofluorescin diacetate [56]. Accordingly, benzene causes oxidatively DNA damage in vitro and in vivo [19]. Benzene and its metabolites have also been shown to inhibit the action of topoisomerase II [41,42] that could result in DNA strand breaks. In summary, both, oxidative DNA damage, and/or topoiomerase II inhibition could be preceding steps in induction of DEL recombination

by benzene and its metabolites. These results further emphasize the importance of genome rearrangement tests in the identification of carcinogens.

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